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Genetic Diversity of *Campylobacter* on Broiler Carcasses Collected Preevisceration and Postchill in 17 U.S. Poultry Processing Plants†

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ABSTRACT

Campylobacter jejuni and *Campylobacter coli* are the most important human enteropathogens among the campylobacters. The objective of this study was to determine how diversity in *Campylobacter* populations found on chicken carcasses collected from 17 broiler processing plants in the United States is impacted by processing. Genetic diversity was determined for up to four isolates per carcass by sequencing the short variable region (SVR) of the *flaA* locus. On 70% of *Campylobacter*-positive carcasses, all isolates were indistinguishable by *flaA* SVR typing. The genetic diversity of *Campylobacter* decreased as carcasses proceeded through processing; *Campylobacter* populations obtained early in processing where carcasses are moved from the kill line to the evisceration line (rehang) were significantly more genetically diverse ($P < 0.05$) than those from carcasses sampled postchill (diversity indices of 0.9472 and 0.9235, respectively). Certain *Campylobacter* subtypes were found only at rehang and not at postchill. Other subtypes were found at postchill and not at rehang. These data suggest that some subtypes may not be able to survive processing, whereas others may persist on the carcass or within the equipment despite stressors encountered in the processing environment.

Campylobacter jejuni and *Campylobacter coli* are the most important human enteropathogens among the campylobacters, affecting an estimated 2.4 million people each year in the United States *(4, 11)*. Risks for contracting campylobacteriosis include consumption and handling of raw or undercooked poultry, cross-contamination from other foods, and consumption of contaminated milk and water *(2, 6, 12, 20, 22)*. *C. jejuni* and *C. coli* can be found on up to 90% of poultry in the United States *(22)* and more than 90% of poultry in Europe, depending on the country *(16)*.

Cross-contamination from a *Campylobacter*-positive flock to a negative flock via contaminated equipment and water can occur during processing *(5, 15)* and is very difficult to control. Finished products that are heavily contaminated with high numbers of microbes are considered undesirable from a food safety and quality point of view. Various processing steps such as chilling and multiple washes are designed to eliminate or control these microbes. In some instances, chemical processing aids are used to further reduce the microbial load on chicken carcasses by removing surface contamination.

Microbial ecology data can be used in the development of strategies to control transmission and to clarify sources of contamination in food processing *(16, 18, 23)*. Molecular subtyping is an important tool for these studies. It allows researchers to trace sources and routes of transmission and identify and monitor specific strains over time and at different sites. Microbial diversity can be defined as a measure of the variation within a population and is not sensitive to whether all the types in a population are known. Sample groups with different indices of diversity indicate that the samples came from different populations or that there is subdivision of the population. It is unclear at present how processing impacts the diversity of *Campylobacter* subtypes on broiler carcasses.

The objective of this study was to examine the genetic diversity of *Campylobacter* populations from broiler carcasses early in processing and compare that to the diversity encountered on fully processed carcasses from the same flock. The short variable region (SVR) of the flagellin locus was analyzed from *Campylobacter* detected on carcasses at rehang and postchill in 17 commercial broiler processing plants. We hypothesized that the diversity in *Campylobacter* populations declines as carcasses move toward the end of processing and that the population diversity is not affected by the choice of chemicals used in processing.

MATERIALS AND METHODS

Origin of isolates. *Campylobacter* isolates used in this study were collected in an earlier study *(7)* in which carcasses were examined from 20 commercial processing plants early in processing (at the rehang station after feather and head removal but before evisceration) and immediately after leaving the chill tank. Ten carcasses from the same flock were collected at both sites in each of four replications; each replication was conducted in a different season. Carcass rinses were cultured for *Campylobacter,* and up to four isolates (as many as were available on the plate, with a

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maximum of four) from each *Campylobacter*-positive carcass were selected and frozen at −80°C in buffered peptone water with 10% sterile glycerol. *Campylobacter* was not detected on carcasses from all 20 plants sampled *(7).* Some plants processed *Campylobacter*-positive flocks on some but not all of the four sample days and some carcass rinses produced fewer than four isolates. As a result, 1,478 *Campylobacter* isolates from 35 unique flocks sampled at 17 processing plants were included in the current study.

DNA extraction. Isolates were revived from frozen storage, streaked for isolation on tryptic soy agar (TSA; Remel, Inc., Lenexa, KS) with 5% sheep blood, and incubated in a sealable bag flushed with microaerobic gas (5% O_2, 10% CO_2, 85% N_2) at 42°C for 24 to 36 h. An isolated colony chosen for subculture was spread for a lawn on TSA, incubated for 24 h in the microaerobic atmosphere, and used in DNA extraction. DNA extraction was performed with a commercial isolation kit (Puregene, Gentra Systems, Minneapolis, MN) following the manufacturer's instructions.

PCR for species identification. An automated PCR system (BAX, DuPont Qualicon, Wilmington, DE) was used to identify the species of the isolates as *C. jejuni* or *C. coli,* as previously described *(10).* Reference strains for *Campylobacter* were *C. jejuni* ATCC 33560 and *C. coli* ATCC 33559. For the DNA samples that did not produce identifiable product during electrophoresis, a multiplex PCR assay was used as described by Wang et al. *(23).*

Amplification and sequencing of *flaA* gene. The *flaA*-SVR PCR was performed as described by Meinersmann et al. *(13)* with primers FLA4F (5′ GGATTTCGTATTAACACAAATGGTGC 3′) and FLA625RU (5′ CAAGWCCTGTTCCWACTGAAG 3′) and a PCR program of 94°C for 2 min and then 30 cycles of 94°C for 15 s, 50°C for 30 s, and 72°C for 1 min. Samples were incubated at 72°C for 2 min and held at 4°C until processed.

Amplification products were analyzed by electrophoresis at 130 V for 60 min using 1× TBE (0.89 M Tris borate, 0.02 M EDTA) running buffer on 2% agarose gels (Seakem LE agarose, Cambrex Bio Science Rockland, Inc., Rockland, ME). Gels were stained with 10 mg/ml ethidium bromide solution (Sigma, St. Louis, MO) and visualized on a UV gel documentation system. PCR products were cleaned using a Qiagen BioRobot and associated QIAquick BioRobot kit (Qiagen, Inc., Valencia, CA).

Cleaned PCR product was sent to the Integrated Biomolecular Resources Laboratory (U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Core Technologies, Wyndmoor, PA) for dye-terminator DNA sequencing using the Applied Biosystems 3730 DNA sequencer and 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) with the FLA4F and FLA625RU primers. Sequence data were assembled with Sequencher 4.7 (Gene Codes Corporation, Inc., Ann Arbor, MI). PAUP version 4.0b10 (Sinauer Associates, Inc., Sunderland, MA) was used to generate distance matrices.

Statistical analysis. The statistical analysis was carried out using the Community Analysis Package 3.0, Species Diversity and Richness (2004, Pisces Conservation, Ltd., Lymington, UK) to find Simpson's index: $D = \Sigma n_i(n_i - 1)/[N(N - 1)]$, where n_i is the number of individuals of species i and $N = \Sigma n_i$. The significance of the differences was determined with Solow's randomization test *(21).* Values of D closer to 1 indicate a greater diversity of subtypes among the population, and a score of 0 indicates that the strains were indistinguishable by the subtyping method used. For the parameters of this study, genetic diversity indicates the probability that if we randomly choose two individuals from a population, they will belong to distinct *flaA*-SVR types, i.e., they have an allele that differs from that of any other subtype by at least 1 bp. Data were analyzed within and across plants, seasons, and sites. Significance was assigned at $P < 0.05$.

Method for naming types. Nucleotide and peptide sequences for alleles of *Campylobacter* FlaA SVR have been collected on a curated database. The current study made use of the *C. jejuni* typing website (http://hercules.medawar.ox.ac.uk/flaA/) developed by Keith Jolley and Man-Suen Chan and maintained at the University of Oxford (Oxford, UK). Each type detected in the current study was entered into the database to determine whether its *flaA*-SVR sequence was already published. Those types that were found in the database were identified by the published number. When a type was not found in the database, its closest sequence match was the basis of the working name. For example, in this study four sequences were closely related to the published sequence of *flaA*-SVR type 54; these sequences were given the names 54.1, 54.2, 54.3, and 54.4.

RESULTS

Campylobacter prevalence on broiler carcasses at both sampling sites (rehang and postchill) and all plants was reported in the original study *(7).* In the current study, 1,478 isolates were identified to species by an automated PCR method. These isolates were from 407 carcasses from 35 flocks that were sampled in 17 plants. *C. jejuni* was the only species detected on carcasses from 21 flocks, and *C. jejuni* and *C. coli* were detected on carcasses from 13 flocks (Table 1). In the spring replication, one flock (from plant 14) was contaminated with only *C. coli* and two flocks (from plants 5 and 11) were contaminated primarily with *C. coli.*

Some isolates died in frozen storage or did not give a product in the *flaA*-SVR PCR assay. For 26 isolates, the *flaA*-SVR PCR assay produced product but the automated species PCR assay did not. Therefore, 1,312 isolates were typed by sequencing the *flaA*-SVR, resulting in identification of 69 *flaA*-SVR types (Fig. 1). *Campylobacter* isolates from plant 8 were the most diverse, with 16 types, whereas plant 3 had the least diversity, with one type (Table 2). More *Campylobacter* isolates were detected and subtyped in samples from plant 8 (129 isolates) than from samples from plant 3 (46 isolates), but a relationship between the number of isolates and diversity was not found (Table 2). Because plant 3 had no diversity among the *Campylobacter* isolates, it was used as the basis for comparison to find significant differences in all diversity indices for C*ampylobacter* from carcasses collected at rehang and postchill (Table 2). The six plants with the lowest diversity (plants 2, 3, 6, 9, 11, and 18) were significantly less diverse ($P < 0.05$) than were the other 11 plants.

Samples collected in the fall had a diversity index of 0.71. More genetic diversity was noted in *Campylobacter* isolates from carcasses collected during winter (0.9195). Isolates from samples collected in spring and summer were not significantly different in their diversity index values (0.8763 and 0.8960, respectively). Within each season, the index of diversity was significantly lower at postchill than at rehang (Table 3). This difference was still evident when the index was recalculated across all seasons and plants.

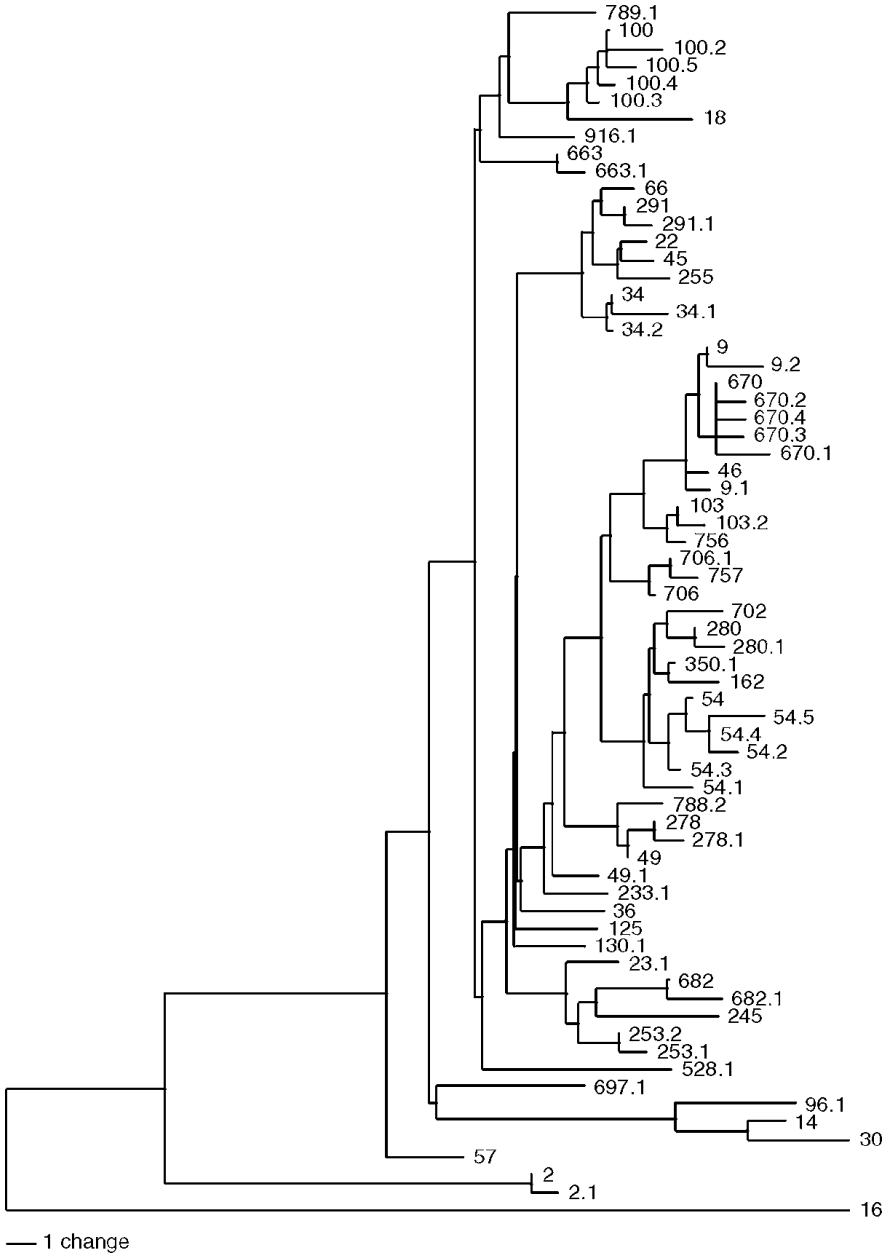

FIGURE 1. *Dendrogram showing relatedness of* Campylobacter flaA-*SVR types (designated by number) detected on broiler carcasses from 17 different commercial processing plants sampled four times in 2005.*

The diversity index of *Campylobacter* isolates from rehang carcasses was 0.9472 and that from postchill carcasses was significantly lower ($P < 0.05$), at 0.9235.

The diversity of *Campylobacter* isolates from individual carcasses is shown in Table 4. Up to four isolates were picked for analysis from each *Campylobacter*-positive carcass. On most carcasses (70%), all isolates were indistinguishable by *flaA*-SVR sequence typing. On only two carcasses (0.5%) were four unique genotypes detected.

The diversity indices of *Campylobacter* isolates from carcasses in plants using different chemical treatments in a postevisceration on-line wash step are shown in Table 5. Except for plants using chlorine dioxide and food-grade acid blend, the diversity of *Campylobacter* on carcasses decreased during processing. The greatest diversity was seen in plants using trisodium phosphate, which was the most commonly used chemical in this study.

The isolation rate of subtypes from carcasses at rehang and postchill is shown in Figure 2. Most subtypes were found more frequently on carcasses early in processing (rehang) rather than later (postchill). Few isolates, most notably subtypes 46 and 14, were found more frequently on carcasses at postchill than at rehang.

DISCUSSION

Understanding how *Campylobacter* is transmitted and contaminates broiler carcasses during processing will help risk managers when they are designing interventions to reduce or eliminate this pathogen in broiler processing plants. Studying diversity within bacterial populations also may help scientists identify bacteria that have become adapted for survival in the processing environment or under various stresses. In this study, we studied two distinct areas of a poultry processing line. In general, *Campylobacter* diversity declined as carcasses moved down the processing line.

Chicken carcasses produced in the United States and

TABLE 1. Campylobacter *species detected on broiler carcasses from each of 17 commercial processing plants sampled in 2005*

Plant no.[a]	Season	No. of isolates[b]	% *C. jejuni*	% *C. coli*
1	Spring	10	100	0
	Summer	16	100	0
2	Spring	34	100	0
3	Fall	43	100	0
4	Fall	42	100	0
	Spring	67	100	0
	Summer	58	96.55	3.45
5	Fall	32	84.38	15.62
	Winter	44	100	0
	Spring	72	2.78	97.22
6	Summer	80	100	0
7	Winter	41	100	0
	Spring	16	100	0
	Summer	48	100	0
8	Winter	37	78.38	21.62
	Spring	52	100	0
	Summer	48	83.33	16.67
9	Winter	20	100	0
10	Winter	38	100	0
	Spring	53	100	0
	Summer	39	100	0
11	Spring	61	3.28	96.72
14	Winter	32	100	0
	Spring	59	0	100
	Summer	55	100	0
15	Winter	39	89.74	10.26
	Spring	54	46.3	53.7
	Summer	31	90.32	9.68
16	Winter	11	100	0
	Summer	30	26.67	73.33
17	Winter	40	100	0
	Spring	35	100	0
	Summer	66	22.73	77.27
18	Winter	38	94.74	5.26
20	Summer	37	89.19	10.81

[a] A different flock was sampled in each plant in each season.
[b] Up to four isolates were recovered from each *Campylobacter*-positive carcass.

United Kingdom are frequently contaminated with multiple subtypes of *Campylobacter (9, 11, 18).* In the current study, 30% of the carcasses tested had more than one *flaA*-SVR type.

Plant 3, with a relatively low number of isolates (46), had no diversity, whereas plant 4, with the highest number of isolates (155), had significantly more diversity among its *Campylobacter* isolates. Despite the use of a diversity algorithm that should normalize for sample numbers *(21),* the current data are in accordance with those of Lindmark et al. *(12)* and indicate a possible correlation between high prevalence of *Campylobacter* in a flock and the presence of several genotypes on carcasses from that flock.

Alter et al. *(3)* found that amplified fragment length polymorphism type distribution changes during processing; broad diversity was observed at the beginning of slaughter and only a few closely related *Campylobacter* subtypes

TABLE 2. *Simpson's index of diversity for* Campylobacter *detected on broiler carcasses at rehang and postchill combined from 17 commercial processing plants over all seasons*

Plant no.	Index of diversity[a]	Total no. of isolates
3	0.000 A	46
6	0.0282 A	70
18	0.0714 A	28
11	0.2361 A	63
2	0.2390 A	31
9	0.3983 A	22
7	0.4517 B	62
1	0.4629 B	29
16	0.5299 B	50
4	0.5442 B	155
14	0.6796 B	86
17	0.7672 B	148
15	0.7746 B	114
20	0.7748 B	35
5	0.7822 B	120
10	0.7904 B	124
8	0.8591 B	129

[a] Diversity values with different letters are significantly different (randomization test, $P \leq 0.05$).

were present after a 24-h chilling period. In the current study in which all plants used an ice water immersion chill of 1 to 2 h, many plants had very low *Campylobacter* prevalence on postchill carcasses, which may affect diversity. Nevertheless, like Alter et al. *(3)* we found overall that processing appeared to lessen *Campylobacter* diversity. However, it is unclear whether the types found late in processing were very closely related because SVR data alone cannot track recombination events that may obscure relatedness.

Other researchers have found that carcasses sampled toward the end of the processing line have been contaminated with campylobacters even when the bacteria were not isolated from the chickens upon arrival at the abattoir *(1, 8, 14, 17).* Subtypes of campylobacters found on carcasses of colonized chicks are not always those most prevalent in the guts of birds *(1, 17).* In the current study, several *Campylobacter* subtypes were detected postchill that were not

TABLE 3. *Simpson's index of diversity for* Campylobacter *detected on broiler carcasses during each season at rehang and postchill sites, all processing plants combined*

Season	Site	*n*	Simpson's index of diversity[a]
Fall	Rehang	88	0.7684 A
	Postchill	30	0.4805 B
Winter	Rehang	295	0.8944 A
	Postchill	46	0.8303 B
Spring	Rehang	287	0.8805 A
	Postchill	127	0.8164 B
Summer	Rehang	311	0.9057 A
	Postchill	128	0.7797 B

[a] Within the same season, diversity indices with different letters are significantly different (randomization test, $P \leq 0.05$).

TABLE 4. *Number of unique* flaA-*SVR types detected per broiler carcass (*n *= 407 carcasses examined)*[a]

No. of types/ carcass	Site	Total no. of carcasses	% total carcasses
1	Rehang	212	52.09
	Postchill	77	18.92
2	Rehang	80	19.66
	Postchill	23	5.65
3	Rehang	11	2.70
	Postchill	2	0.49
4	Rehang	2	0.49
	Postchill	0	

[a] Four or fewer isolates were tested per carcass.

TABLE 5. *Simpson's index of diversity for* Campylobacter *isolates from broiler carcasses separated by carcass treatments at rehang and postchill, all processing plants combined*

Carcass treatment[a]	No. of flocks	Site	Simpson's index of diversity[b]
ASC	5	Rehang	0.8455 A
		Postchill	0.5410 B
CD	2	Rehang	0.5933 A
		Postchill	0.3857 A
FGA	3	Rehang	0.4882 A
		Postchill	0.5779 A
HAS	2	Rehang	0.5631 A
		Postchill	0.0000 B
No treatment	4	Rehang	0.6222 A
		Postchill	0.4962 B
PA	3	Rehang	0.8563 A
		Postchill	0.6445 B
TSP	16	R-hang	0.9306 A
		Postchill	0.8281 B

[a] ASC, acidified sodium chlorite (Sanova, Ecolab Inc., St. Paul, MN); CD, chlorine dioxide (Ashland Specialty Chemical, Boonton, NJ); FGA, blend of food grade acids (FreshFX, SteriFX Inc., Shreveport, LA); HAS, hypochlorous acid system ($Tomco_2$, Tomco Equipment Co., Loganville, GA); PA, peroxyacetic acid–based antimicrobial (Inspexx 100, Ecolab); TSP, trisodium phosphate (Danisco USA Inc., New Century, KS).

[b] Within carcass treatments, diversity indices with different letters are significantly different (randomization test, $P \leq 0.05$).

detected at rehang, which may be indicative of contamination occurring during processing. Processes such as scalding at temperatures near 60°C and chilling in ice water may put selective pressure on *Campylobacter*. Certain stress-resistant subpopulations may survive and ultimately remain in the food chain, indicating that different lineages have different potentials for colonization and for survival when confronted with environmental stressors *(3, 19, 21)*. Many *Campylobacter* types found at the rehang site were not found at postchill. These data suggest that some types are prone to be eliminated or reduced to nonculturable status during processing, whereas other types may survive or even flourish in stressful environments, perhaps persisting on carcasses to retail.

Changes in diversity indicate that subpopulations of *Campylobacter* fare differently in the processing plant, although the present technology does not allow us to discern what characteristics account for the differences. Because of the propensity of *C. jejuni* to engage in lateral gene transfer, the *flaA*-SVR cannot be expected to be linked to the trait that confers the resistance to environmental stressors; we can conclude only that there are resistance traits. More re-

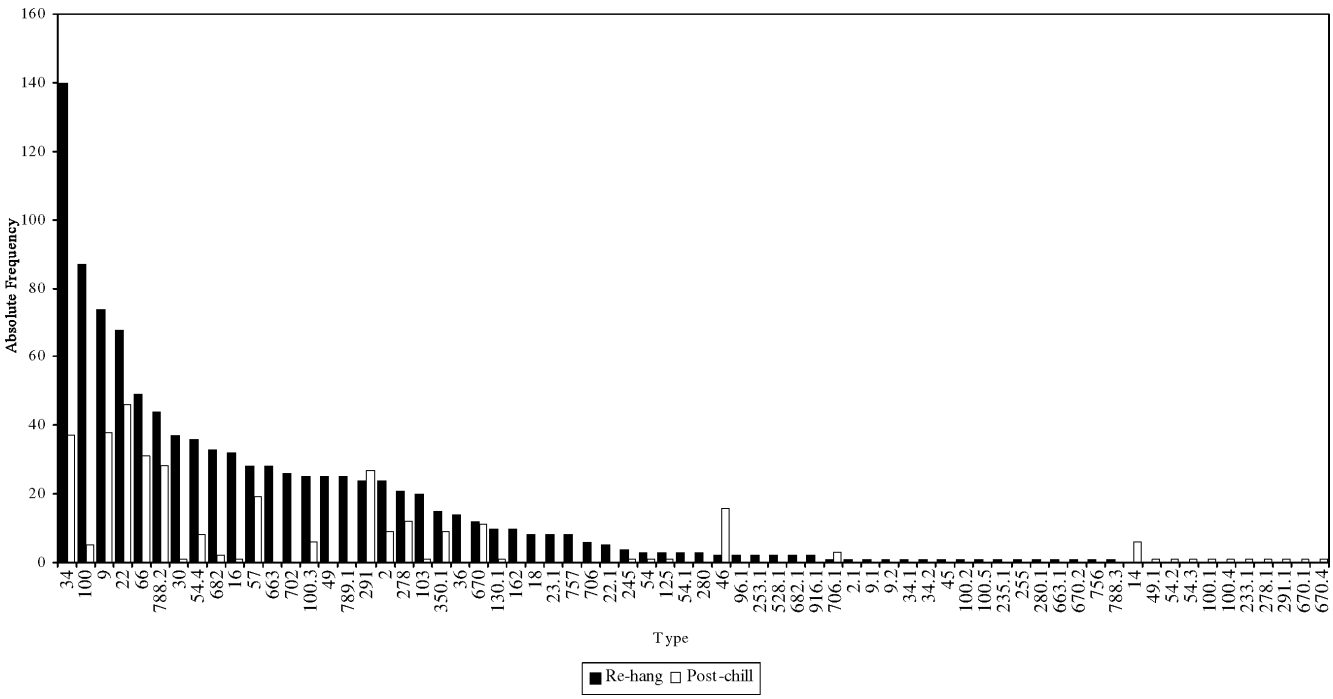

FIGURE 2. *Frequency of* Campylobacter flaA-*SVR types detected across all processing plants and seasons at rehang and postchill sites.*

search is required to study those *Campylobacter* types that are able to survive processing or to proliferate in the processing environment so that any special characteristics involved can be identified.

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